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**UTILITY
PATENT APPLICATION
TRANSMITTAL**

Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No.

2328-111

Total Pages

First Named Inventor or Application Identifier

Ari HINKKANEN

Express Mail Label No.

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

1. ☒ Fee Transmittal Form
(Submit an original, and a duplicate for fee processing)
2. ☒ Specification Total pages [22]
(preferred arrangement set forth below)
 - Descriptive title of the invention
 - Cross references to Related Applications
 - Statement Regarding Fed sponsored R&D
 - Reference to Microfiche Appendix
 - Background of the Invention
 - Brief Summary of the Invention
 - Brief Description of the Drawings
 - Detailed Description
 - Claims
 - Abstract of the Disclosure
3. ☒ Drawing(s) (35 USC 113) (Total Sheets) [12]
4. ☒ Oath or Declaration (Total Pages) [2]
 - a. ☒ Newly executed (original or copy)
 - b. ☐ Copy from a prior application
(37 CFR 1.63(d))
(for continuation/divisional with Box 17 completed)
[Note Box 5 below]
☐ **DELETION OF INVENTOR(S)**
Signed statement attached deleting
inventor(s) named in the prior application,
see 37 CFR 1.63(d)(2) and 1.33(b)
5. ☐ Incorporation by Reference (useable if Box 4b is
checked) The entire disclosure of the prior
application, from which a copy of the oath or
declaration is supplied under Box 4b, is
considered as being part of the disclosure of
the accompanying application and is hereby
incorporated by reference therein.

ADDRESS TO: **Assistant Commissioner of Patents
Box Patent Application
Washington, D.C. 20231**

6. ☐ Microfiche Computer Program (Appendix)
7. Nucleotide and/or Amino Acid Sequence Submission
(if applicable, all necessary)
 - a. ☐ Computer Readable Copy
 - b. ☐ Paper Copy (identical to computer copy)
 - c. ☐ Statement verifying identity of above copies

ACCOMPANYING APPLICATION PARTS

8. ☐ Assignment Papers (cover sheet & document(s))
9. ☐ 37 CFR 3.73(b) Statement
(when there is an assignee)
☒ Associate Power of Attorney
10. ☐ English Translation Document (if applicable)
11. ☐ Information Disclosure Statement /PTO 1449
☐ Copies of IDS Citations
12. ☒ Preliminary Amendment
13. ☒ Return Receipt Postcard (MPEP 503)
(Should be specifically itemized)
14. ☐ Small Entity Statement(s)
☐ Statement Filed in prior application,
Status still proper and desired
15. ☐ Certified Copy of Priority Document(s).
(if foreign priority is claimed)
16. ☐ Other:

17. If a CONTINUING APPLICATION, check appropriate box and supply the requisite information:

☐ Continuation ☐ Divisional ☐ Continuation-in-part (CIP) of prior application No.:

18. CORRESPONDENCE ADDRESS

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15-21-98

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of)
Ari HINKKANEN)
Serial No. (to be assigned)) Examiner:
Filed: 29 January 1998) Group Art Unit:
For: A NEW FUSION PROTEIN AND)
ITS USE IN AN IMMUNOASSAY)
FOR THE SIMULTANEOUS)
DETECTION OF AUTOANTIBODIES)
RELATED TO INSULIN-DEPENDENT)
DIABETES MELLITUS)

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

Prior to initial examination of the above application, filed concurrently herewith, please enter the following amendments:

IN THE ABSTRACT:

At the top of the unnumbered page containing the Abstract, please insert -- 22 --.

IN THE CLAIMS:

Please amend the claims as follows:

In claim 9, line 1, delete "or 8".

Please add the following new claim:

--17. A vector comprising the cDNA according to claim 8.--

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REMARKS

The above amendments to the claims are to delete a multiple dependency, and bring the claims more in line with U.S. practice. The amendment to the Abstract is merely to insert an identifying page number.

It is believed that these amendments do not constitute the insertion of new matter. Prompt consideration of this Preliminary Amendment is requested.

Respectfully submitted,

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Dated: 29 January 1998

A NEW FUSION PROTEIN AND ITS USE IN AN IMMUNOASSAY FOR THE
SIMULTANEOUS DETECTION OF AUTOANTIBODIES RELATED TO
INSULIN-DEPENDENT DIABETES MELLITUS

FIELD OF THE INVENTION

This invention relates to a new fusion protein, its cDNA,
and a vector and a cell comprising said cDNA. Furthermore,
this invention relates to the use of said fusion protein in
an immunoassay for simultaneous detection of autoantibodies
5 related to insulin dependent diabetes mellitus.

BACKGROUND OF THE INVENTION

The publications and other materials used herein to
illuminate the background of the invention, and in
particular, cases to provide additional details respecting
10 the practice, are incorporated by reference.

GAD65, IA2 and insulin are pancreatic proteins produced by
the beta cells (for review see Atkinson and Maclaren 1993).
Autoantibodies to these proteins are detected in patients
with insulin-dependent diabetes mellitus (IDDM) and healthy
15 individuals at risk for developing the disease. More than
80 % of newly-diagnosed IDDM patients have antibodies
against at least one of these proteins (Baekkeskov et al.
1982). The risk of diabetes in relatives of IDDM patients
increases markedly when the number of autoantibodies
20 detected in the serum increases (Bingley et al. 1994; Verge
et al. 1994). In a group of high genetic risk, presence in
serum of antibodies to one or more of these autoantigens
predicted the disease onset accurately (Verge et al. 1996).
Also permanently healthy subjects (as regards IDDM) may
25 have temporarily or permanently antibodies against one of
the three antigens, but antibodies against multiple
antigens occur extremely rarely. It is therefore sought to
simultaneously determine reactivity against two or all
three of the proteins, as the positivity for more than one

GAD65 (Bu et al. 1992) has several epitopes recognised by autoantibodies (Falorni et al. 1996). These are located mostly at the center and C-terminus of the molecule whereas the N-terminal quarter of the molecule is thought to contribute to membrane docking of the protein, and to contain few if any IDDM-informative epitopes (Falorni et al. 1996).

Insulin (Bell et al. 1980) is made by pancreatic β -cells as
20 a precursor preproinsulin which is cleaved to proinsulin.
The proinsulin is further processed to give the insulin
consisting of A and B chains connected together with two
disulphide bridges.

In addition to linear epitopes, autoantibodies are thought to recognize important conformational epitopes resulting

from the three-dimensional structure of the protein (Kim et al. 1993). Antigen molecules produced or assayed using techniques which destroy these structures are less informative as regards IDDM or prediabetes.

- 5 Several methods for detection of autoantibodies in IDDM sera have been elaborated. One method exploits in vitro transcription-translation for producing radioactively labeled autoantigen (IA2, GAD65) (Petersen et al. 1994), while in another method biotin-labeled GAD65 is added to
 10 the patient sera and after formation of immune complexes, free label is detected and quantitated (Mehta et al. 1996). These methods all suffer from suboptimal niveau of informativity, as they employ only one specific autoantigen. Moreover they have the drawbacks associated
 15 with the use of radiochemicals.

- Using a protein molecule in which a combination of the epitopes from at least two but preferably three different autoantigens are represented should detect a larger panel of autoantibodies thus revealing more specifically the
 20 population of individuals at risk of developing the disease.

SUMMARY OF THE INVENTION

- According to one aspect, this invention relates to a new fusion protein having epitopes of at least two of the
 25 autoantigens glutamic acid decarboxylase (GAD65), islet cell antigen (IA2) and preproinsulin (PPINS) wherein said epitopes are connected with a linker peptide, said fusion protein being able to bind to a solid phase.

- According to another aspect, the invention concerns a cDNA
 30 sequence encoding the said fusion protein.

According to a third aspect, the invention concerns a vector and a cell comprising said cDNA.

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According to a fourth aspect, the invention concerns an immunoassay for the simultaneous determination in a sample of a person's body fluid of at least two insulin-dependent diabetes mellitus (IDDM) -related autoantibodies, wherein

5 each autoantibody is specific for an epitope of the autoantigens glutamic acid decarboxylase (GAD65), islet cell antigen (IA2) or preproinsulin (PPINS). The immunoassay comprises the steps of

- incubating said sample with said autoantigens or,

10 alternatively, with the fusion protein according to this invention, said autoantigens or said fusion protein being bound to a solid support,

- adding at least one labeled reagent capable of binding to one or more of said autoantibodies, and

15 - quantifying the signals from the labels bound to the solid phase.

According to still one aspect, the invention concerns a method for diagnosing a person's risk of developing insulin-dependent diabetes mellitus (IDDM), said method

20 comprising the determination in a sample of said person's body fluid of at least two insulin dependent diabetes mellitus (IDDM) -related autoantibodies specific for an epitope of the autoantigens glutamic acid decarboxylase (GAD65), islet cell antigen (IA2) or preproinsulin (PPINS),

25 wherein the presence of at least two of said autoantibodies are indicative for said person's risk of developing IDDM. The order of appearance of these autoantibodies is used to predict the time point of onset of the disease.

BRIEF DESCRIPTION OF THE DRAWINGS

30 Figures 1a and 1b show the cDNA construct for a fusion protein according to this invention,

Figure 2a shows the amino acid sequence of the IA2 protein,

Figure 2b shows the amino acid sequence of the GAD65

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protein,

Figure 2c shows the amino acid sequence of preproinsulin (PPINS),

Figures 3a-3b show the nucleotide sequence encoding GAD65,

5 Figures 3c-3e show the nucleotide sequence encoding IA2,

Figures 3f-3i show the human insulin gene,

Figure 4 shows the fusion protein according to this invention attached to a solid support, autoantibodies attached to epitopes of said protein, and labeled reagents
10 bound to said autoantibodies, wherein the reagents are labeled with different labels, and

Figure 5 shows the fusion protein according to this invention attached to a solid support, autoantibodies attached to epitopes of said protein, and labeled reagents
15 bound to said autoantibodies, wherein the reagents are labeled with the same label.

DETAILED DESCRIPTION OF THE INVENTION

The term "epitope" can be an amino acid sequence anything from very few (about 5 to 10) amino acids of the
20 autoantigens up to the whole autoantigen. Preferable lengths of the epitopes are represented by the underlined amino acid sequences in Figures 2a and 2b, and the whole antigen sequence is disclosed in Figure 2c. Thus, the epitope of IA2 comprises preferably the amino acids 771-979
25 of the amino acid sequence shown in Figure 2a. Another preferred alternative is the whole intracellular domain (amino acids ranging from about 576 to 979 of the sequence in Figure 2a). The epitope of GAD65 comprises preferably the amino acids 102-585 of the amino acid sequence shown in
30 Figure 2b, and the epitope of PPINS comprises preferably

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all the amino acids 1-110 of the polypeptide shown in Figure 2c. It should be noted that the above mentioned specific sequences are examples only.

According to a preferred embodiment, the fusion protein has 5 epitopes of each of the autoantigens GAD65, IA2 and PPINS. Such a fusion protein allows simultaneous detection of autoantibodies specific for any of said autoantigens.

Said fusion protein containing epitopes of GAD65, IA2 and PPINS is formed by combining these domains via short 10 peptides consisting of amino acid residues, e.g. lysine and arginine residues.

The epitopes from distinct autoantigens will be linked together via short peptides containing e.g. several lysine residues, which allows preferential labeling of these lys- 15 residues. For construction of the polygenic cDNA, the linker-encoding cDNA contains a recognition site for a rarely cutting restriction enzyme such as Not I or Sgf I (see Figure 1a and 1b).

These linker residues may be connected to a member of an 20 affinity binding pair so as to enable the binding of said fusion protein to a solid phase. The bioaffinity pair may be e.g. biotin - streptavidin. The residues (lysine) can be biotinylated after which the fusion protein is attached to a streptavidin-coated solid phase. The solid phase can e.g. 25 be a well of a microtitration strip or plate. Alternatively, the solid phase consists of microparticles.

The fusion protein can alternatively be bound to the solid phase by direct adsorption. Furthermore, the fusion protein can be covalently linked to the solid phase. In this case 30 the fusion protein must be provided with groups able to create a covalent bond with the solid phase.

Figures 2 and 3 show the amino acid sequences and the nucleotide sequences, respectively, of the preferred

epitopes.

The following illustrates the construction of the fusion protein and its preparation.

- 5 The N-terminus of the hybrid protein will contain a flag peptide NH₂-DYKDDDDK-COOH with a free N-terminal amino group to allow recognition of the protein using M1 monoclonal antibody (ATCC cell line nr. HB 9259). This enables detection of the protein in SDS-PAGE where not all
10 monoclonals function.

- At the carboxy-terminal end of the fusion protein and in the single antigens a motif X-X-G-S-H-H-H-H-H is introduced to allow purification of the protein with metal chelate affinity chromatography and detection with
15 monoclonal antibody against this epitope (Cedarlane Laboratories Ltd, Canada).

The GAD65 gene (Bu et al. 1992) is, for example, amplified with PCR (nucleotides 1311-1755) in such a manner that 101 amino acid residues are removed from the N-terminus.

- 20 The 3'-end oligonucleotide contains 17 bases complementary to the mRNA of GAD65 and an additional sequence encoding half of a peptide forming the bridge between GAD65 and IA2 domains.

The nucleotide sequence of the bridge is for example

- 25 Not I
GAD65-AAGAAGAAGCGGCCGCGAAAGAAGAAG-IA2 (amino acid sequence of the peptide KKKRPRKKK), or

- Sfg I
30 GAD65-AAGAAGAAGCGATCGCGAAAGAAGAAG-IA2 (amino acid sequence KKKRSRKKK). The restriction enzyme recognition sites are underlined in the middle. The fragments are made from a

plasmid harbouring said cDNAs with PCR and digested with appropriate restriction enzymes (e.g. Not I or Sfg I) and cloned into appropriate vectors. The GAD65 part is linked to IA2 and this to PPINS, using general cloning techniques.

- 5 The PPINS gene 5'-oligo contains half of the polylysine-
arginine-encoding sequence with a Not I or Sfg I site for
coupling to the IA2 gene 3'-end. The 3'-oligo of PPINS has
a histidine hexapeptide-encoding sequence to enable
antibody recognition and metal chelate chromatography
10 purification and/or immobilization if necessary (Mauch et
al. 1993).

Purified, restriction enzyme-treated PCR fragments are cloned in a FastBac derivative and E.coli DH10Bac cells are transfected with the plasmid. Recombinant clones are

- 15 selected and DNA isolated and transfected into Sf9 insect
cells.

Virus-producing cells are cultivated and stock virus made. Large-scale cultures are used to produce recombinant single proteins and the polyprotein.

- 20 SDS-PAGE/Western analysis is used to analyse size and immunoreactivity of the recombinant polyproteins. The proteins are blotted onto a nitrocellulose or nylon membrane and GAD/IA2/PPINS antibodies used to detect the product visualised with enhanced chemiluminescence, ECL.
- 25 For purification of the polyprotein GAD65-specific monoclonal antibody (GAD6, Developmental Studies Hybridoma Bank, Iowa University) is immobilized to Sepharose 4B activated with cyanogen bromide (Pharmacia, Uppsala, Sweden). Elution of the protein is performed at low pH (3-
- 30 4) and solubility is achieved by adding detergents (e.g. Nonidet or Tween) to allow dissociation from the membranes.

The steps from cloning to large scale production can be

described in more detail as follows:

1. Cloning into the pK503-9 vector (Kari Keinänen VTT Finland), a derivative of pFastBac (Gibco BRL Paisley Scotland) of GAD65, or IA2 or PPINS gene, each containing a
 5 flag recognition signal (FLAG^R, Immunex Corporation) for antibody detection and a signal peptide for ecdysone glucotransferase (EGT) for transport into the endoplasmatic reticulum for removal of the signal peptide with simultaneous release of N-terminal aspartate for M1
 10 antibody recognition. The constructs contain each a X-X-G-S-H-H-H-H-H carboxyterminal peptide to allow metal chelate affinity purification and detection with specific antibody (Cedarlane, Canada) of the product.
2. Transformation into competent E. coli DH10Bac cells of
 15 the plasmids containing the single genes.
3. Isolation of recombinant Bacmid DNA and transfection with the fused DNA of the Sf9 or Hi-5 insect cells.
4. Production of recombinant stock virus.
5. Large scale production of the proteins.
- 20 6. Cloning into pK503-9 vector of a cDNA construct for the fusion protein (FP) comprising GAD65 (nt 1311-1755; aa 102-585)-IA2(nt 2313-2937; aa 771-979)-PPINS (nt 2424-2610 and 3396-3539 (of the genomic DNA sequence, accession No. V00565); aa 1-110) in all alternative orders.
- 25 7. Transformation into competent E. coli DH10Bac cells of the plasmids containing the fusion protein.
8. Isolation of recombinant Bacmid DNA and transfection with the fused DNA of the Sf9 or Hi-5 insect cells.
9. Production of recombinant stock virus.

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10. Large scale production of the fusion protein.

In case the baculovirus expression system does not work optimally, alternative systems such as E.coli, yeast, or in vitro transcription translation assay (Petersen et al. 1994) will be used for production of said polypeptides.

The present invention relates further to the use of the fusion protein in an immunoassay for the detection of several pancreatic beta-cell autoantibodies in IDDM patients and prediabetic sera. The assay may detect patients at risk of developing IDDM, i.e. having a pre-IDDM condition. As a multicomponent assay, the method could also be used to predict the time point of onset of the disease. The methodology which combines epitopes of several islet beta cell autoantigens increases the informativity and prediction value of the test aimed at prediction of risk and onset of disease in individuals genetically predisposed to IDDM.

In the immunoassay according to this invention, a sample of the person's body fluid (e.g. serum) is incubated with the fusion protein bound to a solid surface, e.g. a microtitration plate. The bound autoantigens are thereafter detected with a labeled reagent. The reagents can be the single autoantigens GAD65, IA2 and PPINS; or proteins comprising epitopes thereof. These reagents are used to detect free antigen-binding regions (V-regions) on the bound autoantibodies. One variant of the method will be used for differential detection of the individual autoantigen specificities of the antibody in one assay if individual autoantigens (AAGs) labeled with three different labels are used (see Figure 4). Alternatively, when the polyprotein (the fusion protein) is labeled with only one label, it can be used to reveal the sum of these three reactivities in the sample (Figure 5). The same result is achieved if the single antigens are all labeled with the same label. The labeled reagent can further be an anti-

human monoclonal antibody. In this case the assay can reveal only the sum of the three autoantibodies.

The technique which involves use of the label attached to the fusion protein or individual autoantigens circumvents several problems encountered in the conventional assays. First, there is little or no nonspecific binding to the vials due to the fact that the carrier surfaces have already been blocked with the corresponding antigen. Second, the attachment via a bioaffinity pair such as streptavidin/biotin interaction to the vial and use of a flexible peptide between the individual antigenic epitopes enable free motion and folding of the protein in the solution (Figure 5).

The label can be any suitable label. However, according to a preferred embodiment, the label is a lanthanide. In case three different labels are used, said labels can be e.g. Eu, Sm, Tb and Dy (Siitari et al. 1990; Hemmilä et al. 1993). In such a case the detection is based on time-resolved fluorescence.

- 20 The free labeled reagent can be removed after the incubation step before the signal is quantified (heterogeneous assay), or the signal can be quantified without foregoing removal of the free labelled reagent (homogeneous assay).
- 25 The procedures are preferably automatized. Automatization of the procedures involves laboratory robots which apply samples onto cover slips and the fluorescence is detected in an micro array system in an appropriate unit (Wallac OY, Finland).
- 30 The simultaneous detection of antibodies against the three autoantigens increases the capacity to process large sample series. The use of a micro array system substantially increases the capacity. This has become necessary as

nationwide screenings of newborns are undertaken in several research centers.

The test principle using time-resolved fluoroimmunoassay (TR-FIA) offers an extremely sensitive means for detection
 5 of autoantibodies with minimum amount of nonspecific reactivity due to used specific antigen label. The longevity of the lanthanide label is also an advantage as compared to radiolabel.

The system allows retaining of important conformational
 10 epitopes of the antigen as immobilization of the polyprotein is via specific flexible intervening sequences and causes minimal torsion to the antigen.

The following illustrates the use of the fusion protein in an immunoassay:

15 To the polyprotein (fusion protein) biotin is bound in limiting conditions to prevent other than the lysine residues of the linker peptide to be biotinylated. Streptavidine-coated microscope slides are treated with biotin - fusion protein and the residual sites are blocked
 20 with bovine serum albumin or another suitable binding protein.

M1 flag-specific monoclonal antibody will be used to monitor binding onto solid support of free recombinant autoantigens while autoantigen-specific monoclonals (e.g.
 25 GAD1, GAD6, MICA-3 (Boehringer) etc.) will be used to detect availability of specific epitopes. After incubation with sample sera, Eu-labeled GAD65, Sm-labeled IA2 and Tb-labeled PPINS (produced as a single protein with the baculosystem) are printed robotically onto the microscope
 30 slides in four quadrants covering an area of about 1 cm², allowed to bind, washed and dried in vacuum, and the fluorescence is measured on TR fluorometer.

For specificity testing recombinant GAD65, IA2 and PPINS,
5 or fusion protein are added into patient sample to
preadsorb specific antibodies.

The high density array system is fully automatized.

Example 1

Isothiocyanatophenyl-DTTA-Eu, or Tb, or Sm (Mukkala 1989) will be used for labeling of the FP or the single autoantigens. Mainly the protocols of Lövgren & Pettersson (1990) and Hemmilä et al. (1984) will be followed. 30-100 fold molar excess of the label substance will be used giving approximately 10-12 lanthanide molecules per protein molecule. For Tb, 500 fold excess will be used. The coupling is carried out for 18 hr at 0 °C in 0.1 M bicarbonate buffer pH 9.2. The Eu (Tb,Sm)-AAG complex is separated from free Eu (Tb, Sm) by gel filtration in a Sepharose 6B column equilibrated with 0.05 M Tris-HCl buffer pH 7.75 containing 0.9% NaCl and 0.05% NaN₃. The Eu-AAG complex is stored at 4 °C.

Example 2**Immunoassay**

The assay is performed in the wells of polystyrene microtitration strip coated with unlabeled autoantigen
5 prepare for 18 hr at 25 °C in 0.1 M bicarbonate buffer pH 9.6 (Siitari & Kurppa 1987). The strips are washed prior to use with 0.9% NaCl containing 0.05 % Tween 20 and 0.3% Germal II. To each well 100 µl of diluted (1:10) serum is added and incubated for 1 hr at 40 °C, washed 2x with the
10 wash solution and 200 µl of the Eu-labeled autoantigen fraction (50 ng/well) is added.

The strips are incubated for 1 hr at 40 °C. The strips are washed 5x with the washing solution. Thereafter Enhancement Solution (EG&G Wallac) 200 µl/well is added. Strips are
15 shaken for 10 min in a plate shaker and measured in EG&G Wallac Victor fluorometer for 1s/specimen. The photons emitted are measured as counts/s. Automated data reduction program calculates mean value of duplicates and the coefficient of variation (CV%).

20 For future development, the assay formate will be miniaturized e.g. by immobilizing the autoantigen molecules onto microparticles (Lövgren et al. 1997) or as a microarray onto glass cover slips.

It will be appreciated that the methods of the present
25 invention can be incorporated in the form of a variety of embodiments, only a few of which are disclosed herein. It will be apparent for the specialist in the field that other embodiments exist and do not depart from the spirit of the invention. Thus, the described embodiments are illustrative
30 and should not be construed as restrictive.

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2025 RELEASE UNDER E.O. 14176

CLAIMS

1. A fusion protein having epitopes of at least two of the autoantigens glutamic acid decarboxylase (GAD65), islet cell antigen (IA2) and preproinsulin (PPINS) wherein said epitopes are connected with a linker peptide, said fusion
5 protein being able to bind to a solid phase.
2. The fusion protein according to claim 1 having epitopes of each of the autoantigens GAD65, IA2 and PPINS.
3. The fusion protein according to claim 2 wherein
- the epitope of IA2 comprises the amino acids 771-979 of
10 the amino acid sequence shown in Figure 2a,
- the epitope of GAD65 comprises the amino acids 102-585 of the amino acid sequence shown in Figure 2b, and
- the epitope of PPINS comprises all the amino acids 1-110 of the amino acid sequence shown in Figure 2c.
- 15 4. The fusion protein according to claim 1 wherein the linker peptide comprises lysine and argine residues.
5. The fusion protein according to claim 4 wherein said linker peptide is provided with a member of an affinity binding pair so as to enable the binding of said fusion
20 protein to the solid phase.
6. The fusion protein according to claim 5 wherein the affinity binding pair is biotin - streptavidin.
7. A cDNA encoding the fusion protein according to claim 1 wherein said cDNA comprises the nucleotide sequences
25 encoding the epitopes of at least two of the autoantigens glutamic acid decarboxylase (GAD65), islet cell antigen (IA2) and preproinsulin (PPINS).
8. A cDNA encoding the fusion protein according to claim 3

005570 6657060

a

5

c) nucleotides 2424 to 2610 and 3397 to 3539 of the sequence according to Figure 3f-3i encoding PPINS, aa 1-110, where said nucleotide sequences a), b) and c) can appear in any relative order.

10. An *E. coli* cell encompassing the cDNA according to claim 7.

20 - incubating said sample with a fusion protein according to
claim 1, said fusion protein being bound to a solid
support,

25 - quantifying the signals from the labels bound to the solid phase.

12. The immunoassay according to claim 11 wherein the labeled reagent is an anti-human monoclonal antibody.

13. The immunoassay according to claim 11 wherein the
30 labeled reagent comprises at least two antigens labeled
with different labels, each antigen being one of the
autoantigens GAD65, IA2 or PPINS; or proteins comprising
epitopes thereof.

5 GAD65, IA2 or PPINS; or proteins comprising epitopes thereof.

15. The immunoassay according to claim 11 wherein the label is a fluorescent lanthanide chelate.

Adel R 17

(57) ABSTRACT

The invention relates to a fusion protein having epitopes of at least two of the autoantigens glutamic acid decarboxylase (GAD65), islet cell antigen (IA2) and preproinsulin (PPINS) wherein said epitopes are connected with a linker peptide. The fusion protein must be able to bind to a solid phase.

The invention also concerns the cDNA, and a vector and cell comprising said cDNA. Furthermore, this invention relates to the use of said fusion protein in an immunoassay for the simultaneous detection of autoantibodies related to insulin-dependent diabetes mellitus.

00015399 01299
0000210 00000000

| | |
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| APPROVED | O.G.FIG. |
| BY | CLASS SUBCLASS |
| DRAFTSMAN | |

Flag-peptide GAD65 Sgf I IA2 Sgf I PPINS poly-his
 DYKDDDDK-----KKKRPRKKK-----KKKRPRKKK-----CNGSHHHHHH

FIG. 1a

Flag-peptide GAD65 Not I IA2 Not I PPINS poly-his
 DYKDDDDK-----KKKRSRKKK-----KKKRSRKKK-----CNGSHHHHHH

FIG. 1b

| | |
|-----------|-----------|
| APPROVED | O.G. FIG. |
| BY | CLASS |
| DRAFTSMAN | SUBCLASS |

IA2 Underlined aa 771-979 Accession No. L18983

MRRPRRPGGLGGGLRLLCLLLSSRPGGCSA VSAHGCLFDRRLCSHLEVCIQDGLFGQCQVGVQARPLLQVTSPVLQRL
QGVLRQLMSQGLSWHDDL TQYVISQEMERIPRLRPEPRDRSGLAPKRPGPAGELL LQDIPTGSAPAAQHRLPQPPVGKGG
AGASSLSPLQAE LPPLEHLLPPQPPHPSLSYEPALLQPYLFHQFGSRDGSRVSESGPMVSVGPLPKAEAPALFSRTASKGI
FGDHPGHSYGDLPGPSAQLFQDSGLLYLAQELPAPSRARVPRLPEQGSSRAEDSPEGYEKEGLGDRGEKPASPAVQPDAAAL
QRLAAVLAGYGVELRQLTPEQLSTLLTLLQLLPKAGRNPGGVVNVGADIKKMEGPVEGRDTAELPARTSPMPGHPTASPT
SSEVQQVPSVSSEPPKAA RPPVTPVLEKKSPLGQSQPTVAGQPSARPA AEEYGYIVTDQKPLSLAAGVKLLLEILAEHVHMSS
GSFNISVVGPALTFRIRHNEQNLSLADVTQAGLVKSELEAQTGLQILQTGVGQREEAAVLPQTAHSTSPMRSVLLTLVALA
GVAGLLVALAVALCVRHARQQDKERLAALGPEGAGHDTTFEYQDLCRQHMA TKSLFNRAEGPEPSRVSSVSQFSDDAAQ
ASPSHSSTPSWCEEP AQANMDISTGHMILAYMEDHLNRDR LAKEWQALCAYQAEPTCAT AQEGEKNKNRHPDFLPYDH
ARIKLKVESPSRSDYINASPIEHDPMPAYIATOGPLSHTIADFQWQVWESGCTVIVMLTPLVEDGVKQCDRYWPDEGASLY
HVYEVNLVSEHIWCEDFLVSFYLNKVVQTOETRTLTOHFLSWPAEGTPASTRPLLDERRKV NKC YRGRSCPIVHCSDGAGR
TGTYILIDMV LNRMAKGVKEIDIAATLEHV RDORPGLVRSKDOFEFALTAVAEVNAILKALPQ

FIG. 2a

GAD65 Underlined aa102-585 Accession No. M74826

MASPGSGFWSFGSDSENPGTARA WCQVAQKFTGGIGNKLCALLYGDAEKPAESGSGQPPRAAARCAACDQKPCS
CSKVDVNYAFLHATDLLPACDGERPTLAFLQDVMNILLQYVVKSEDRSTKVIDFHYPNELLOEYNWELADQPONLEEILMHC
QTTLKYAIKTGHPRYFNQLSTGLDMVGLAADWL TSTANTNMFTYEIAPVFVLL EYVTLK KMR EIIGWPGSGDGFSPGGAIS
NMYAMMIARFKMFPEVKEKGMAALPRLIAFTSEHSHFSLKKGAAALGIGTDSVILKCDERGMIPSDLERRILEAKQKGFVPF
LVSATAGTTVYGAFDPLLA VADICKYKIWMHVDAAWGGGLMSRKKHWKLSGVERANSVTWNP HKMMGVPLQCSALLY
REEGLM QNCNOMHAS YLFOODKHYDLSYDTGDKALOCGRHVDVFKLWLMWR AKGTTGFEAHVDKCLJELAEYLYNIIKNR
EGYEMVFDGKPKOHTNVCFWYIPPSLRTLEDNEERM SRLSKVAPVIKARMMEY GTTMVSYOPLGDKVNFRRMVISNPAATHQ
DIDFLJEEIERLGQDL

Translation Human preproinsulin.
EMBL accession nr. v00565

FIG. 2b

MALWMRLPL LALLALWGPDPAAAFVNQHL CGSHLVEALYL VCGERGFYVT
PKTRREAEDLQVGQVELGGGPGAGSLQPLALEGSLQKR GIVEQCCTSI CSLYQ
LENYCN

FIG. 2c

Human GAD65 nucleotide sequence

M74826 Length: 2457 September 1, 1995 12:22 Type: N Check: 8038 ..

1 ACCCGCCCTC GCCGCTCGGC CCCGCGCGTC CCCGCGCGTG CCCTCCTCCC
51 GCCACACGGC ACGCACGCGC GCGCAGGGCC AAGCCGAGGC AGCCGCCCCG
101 AGCTCGCACT CGCTGGCGAC CTGCTCCAGT CTCCAAAGCC GATGGCATCT
151 CCGGGCTCTG GCTTTTGGTC TTTCGGGTCTG GAAGATGGCT CTGGGGATTG
201 CGAGAATCCC GGCACAGCGC GAGCCTGGTG CCAAGTGGCT CAGAAGTTCA
251 CGGGCGGCAT CGGAAACAAA CTGTGCGCCC TGCTCTACGG AGACGCCGAG
301 AAGCCGGCGG AGAGCGGCGG GAGCCAACCC CCGCGGGCCG CCGCCCGGAA
351 GGCCGCCTGC GCCTGCGACC AGAAGCCCTG CAGCTGCTCC AAAGTGGATG
401 TCAACTACGC GTTCTCCAT GCAACAGACC TGCTGCCGGC GTGTGATGGA
451 GAAAGGCCCA CTTTGGCGTT TCTGCAAGAT GTTATGAACA TTTTACTTCA
501 GTATGTGGTG AAAAGTTTCG ATAGATCAAC CAAAGTGATT GATTTCCATT
551 ATCCTAATGA GCTTCTCCAA GAATATAATT GGGAATTGGC AGACCAACCA
601 CAAAATTTGG AGGAAATTTT GATGCATTGC CAAACAACCT TAAAATATGC
651 AATTAAACAA GGGCATCCTA GATACTTCAA TCAACTTTCT ACTGGTTTGG
701 ATATGGTTGG ATTAGCAGCA GACTGGCTGA CATCAACAGC AAATACTAAC
751 ATGTTACCT ATGAAATTGC TCCAGTATTT GTGCTTTTGG AATATGTCAC
801 ACTAAAGAAA ATGAGAGAAA TCATTGGCTG GCCAGGGGGC TCTGGCGATG
851 GGATATTTTC TCCCGGTGGC GCCATATCTA ACATGTATGC CATGATGATC
901 GCACGCTTTA AGATGTTCCC AGAAGTCAAG GAGAAAGGAA TGGCTGCTCT
951 TCCCAGGCTC ATTGCCTTCA CGTCTGAACA TAGTCATTTT TCTCTCAAGA
1001 AGGGAGCTGC AGCCTTAGGG ATTGGAACAG ACAGCGTGAT TCTGATTAAA
1051 TGTGATGAGA GAGGGAAAAT GATTCCATCT GATCTTGAAA GAAGGATTCT
1101 TGAAGCCAAA CAGAAAGGGT TTGTTCTTTT CCTCGTGAGT GCCACAGCTG
1151 GAACCACCGT GTACGGAGCA TTTGACCCCC TCTTAGCTGT CGCTGACATT
1201 TGCAAAAAGT ATAAGATCTG GATGCATGTG GATGCAGCTT GGGGTGGGGG
1251 ATTACTGATG TCCCGAAAAC ACAAGTGGAA ACTGAGTGGC GTGGAGAGGG

FIG. 3a

APPROVED O.G. FIG.
BY CLASS SUBCLASS
DRAFTSMAN

BBB27D-66ESTD6D

1301 CCAACTCTGT GACGTGGAAT CCACACAAGA TGATGGGAGT CCCTTTGCAG
 1351 TGCTCTGCTC TCCTGGTTAG AGAAGAGGGA TTGATGCAGA ATTGCAACCA
 1401 AATGCATGCC TCCTACCTCT TTCAGCAAGA TAAACATTAT GACCTGTCCT
 1451 ATGACACTGG AGACAAGGCC TTACAGTGCG GACGCCACGT TGATGTTTTT
 1501 AAACATATGGC TGATGTGGAG GGCAAAGGGG ACTACCGGGT TTGAAGCGCA
 1551 TGTTGATAAA TGTTTGGAGT TGGCAGAGTA TTTATACAAC ATCATAAAAA
 1601 ACCGAGAAGG ATATGAGATG GTGTTTGATG GGAAGCCTCA GCACACAAAT
 1651 GTCTGCTTCT GGTACATTCC TCCAAGCTTG CGTACTCTGG AAGACAATGA
 1701 AGAGAGAATG AGTCGCCTCT CGAAGGTGGC TCCAGTGATT AAAGCCAGAA
 1751 TGATGGAGTA TGAACCACA ATGGTCAGCT ACCAACCCTT GGGAGACAAG

 1801 GTCAATTTCT TCCGCATGGT CATCTCAAAC CCAGCGGCAA CTCACCAAGA
 1851 CATTGACTTC CTGATTGAAG AAATAGAACG CTTGGACAA GATTATAAT
 1901 AACCTTGCTC ACCAAGCTGT TCCACTTCTC TAGAGAACAT GCCCTCAGCT
 1951 AAGCCCCCTA CTGAGAAACT TCCTTTGAGA ATTGTGCGAC TTCACAAAAT
 2001 GCAAGGTGAA CACCACTTTG TCTCTGAGAA CAGACGTTAC CAATTATGGA
 2051 GTGTCACCAG CTGCCAAAAT CGTAGGTGTT GGCTCTGCTG GTCACTGGAG
 2101 TAGTTGCTAC TCTTCAGAAT ATGGACAAAG AAGGCACAGG TGTAATATA
 2151 GTAGCAGGAT GAGGAACCTC AAACGGGTA TCATTTGCAC GTGCTCTTCT
 2201 GTTCTCAAAT GCTAAATGCA AACACTGTGT ATTTATTAGT TAGGTGTGCC
 2251 AAACATACCGT TCCCAAATTG GTGTTTCTGA ATGACATCAA CATTCCCCCA
 2301 ACATTACTCC ATTACTAAAG ACAGAAAAAA ATAAAAACAT AAAATATACA
 2351 AACATGTGGC AACCTGTTCT TCCTACCAA TATAAACTTG TGTATGATCC
 2401 AAGTATTTTA TCTGTGTTGT CTCTCTAAAC CCAAATAAAT GTGTAAATGT
 2451 GGACACA

FIG. 3b

| | |
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| APPROVED | O.G. FIG. |
| BY | CLASS SUBCLASS |
| DRAFTSMAN | |

SECRETED

Human IA-2 nucleotide sequence

L18983 Length: 3613 November 20, 1997 16:45 Type: N Check: 6409 ..

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|-----------|----------------|
| APPROVED | O.G.F.G. |
| BY | CLASS SUBCLASS |
| DRAFTSMAN | |

1 CAGCCCCTCT GGCAGGCTCC CGCCAGCGTC GCTGCGGCTC CGGCCCCGGA
51 GCGAGCGCCC GGAGCTCGGA AAGATGCGGC GCCCGCGGCG GCCTGGGGGT
101 CTCGGGGGAT CCGGGGGTCT CCGGCTGCTC CTCTGCCTCC TGCTGCTGAG
151 CAGCCGCCCC GGGGGCTGCA GCGCCGTTAG TGCCACGGC TGTCTATTTG
201 ACCGCAGGCT CTGCTCTCAC CTGGAAGTCT GTATTCAGGA TGGCTTGTTT
251 GGGCAGTGCC AGGTGGGAGT GGGGCAGGCC CGGCCCCTTT TGCAAGTCAC
301 CTCCCCAGTT CTCCAACGCT TACAAGGTGT GCTCCGACAA CTCATGTCCC
351 AAGGATTGTC CTGGCACGAT GACCTCACCC AGTATGTGAT CTCTCAGGAG
401 ATGGAGCGCA TCCCCAGGCT TCGCCCCCA GAGCCCCGTC CAAGGGACAG
451 GTCTGGCTTG GCACCAAGA GACCTGGTCC TGCTGGAGAG CTGCTTTTAC
501 AGGACATCCC CACTGGCTCC GCCCCTGCTG CCCAGCATCG GCTTCCACAA
551 CCACCAAGTG GCAAAGGTGG AGCTGGGGCC AGCTCCTCTC TGTCCCCTCT
601 GCAGGCTGAG CTGCTCCCGC CTCTCTTGGA GCACCTGCTG CTGCCCCCAC
651 AGCCTCCCCA CCCTTCACTG AGTTACGAAC CTGCCTTGCT GCAGCCCTAC
701 CTGTTCCACC AGTTTGGCTC CCGTGATGGC TCCAGGGTCT CAGAGGGGCTC
751 CCCAGGGATG GTCAGTGTCTG GCCCCCTGCC CAAGGCTGAA GCCCCTGCCC
801 TCTTCAGCAG AACTGCCTCC AAGGGCATAT TTGGGGACCA CCCTGGCCAC
851 TCCTACGGGG ACCTTCCAGG GCCTTCACCT GCCCAGCTTT TTCAAGACTC
901 TGGGCTGCTC TATCTGGCCC AGGAGTTGCC AGCACCCAGC AGGGCCAGGG
951 TGCCAAGGCT GCCAGAGCAA GGGAGCAGCA GCCGGGCAGA GGAATCCCCA
1001 GAGGGCTATG AGAAGGAAGG ACTAGGGGAT CGTGGAGAGA AGCCTGCTTC
1051 CCCAGCTGTG CAGCCAGATG CGGCTCTGCA GAGGCTGGCC GCTGTGCTGG
1101 CGGGCTATGG GGTAGAGCTG CGTCAGCTGA CCCCTGAGCA GCTCTCCACA
1151 CTCCTGACCC TGCTGCAGCT ACTGCCCAAG GGTGCAGGAA GAAATCCGGG
1201 AGGGGTTGTA AATGTTGGAG CTGATATCAA GAAAACAATG GAGGGGCCCG
1251 TGGAGGGCAG AGACACAGCA GAGCTTCCAG CCCGCACATC CCCCATGCCT

FIG. 3c

1301 GGACACCCCA CTGCCAGCCC TACCTCCAGT GAAGTCCAGC AGGTGCCAAG
 1351 CCCTGTCTCC TCTGAGCCTC CCAAAGCTGC CAGACCCCCT GTGACACCTG
 1401 TCCTGCTAGA GAAGAAAAGC CCACTGGGCC AGAGCCAGCC CACGGTGGCA
 1451 GGACAGCCCT CAGCCCGCCC AGCAGCAGAG GAATATGGCT ACATCGTCAC
 1501 TGATCAGAAG CCCCTGAGCC TGGCTGCAGG AGTGAAGCTG CTGGAGATCC
 1551 TGGCTGAGCA TGTGCACATG TCCTCAGGCA GCTTCATCAA CATCAGTGTG
 1601 GTGGGACCAG CCCTCACCTT CCGCATCCGG CACAATGAGC AGAACCTGTC
 1651 TTTGGCTGAT GTGACCCAAC AAGCAGGGCT GGTGAAGTCT GAACTGGAAG
 1701 CACAGACAGG GCTCCAAATC TTGCAGACAG GAGTGGGACA GAGGGAGGAG
 1751 GCAGCTGCAG TCCTTCCCCA AACTGCGCAC AGCACCTCAC CCATGCGCTC
 1801 AGTGCTGCTC ACTCTGGTGG CCCTGGCAGG TGTGGCTGGG CTGCTGGTGG
 1851 CTCTGGCTGT GGCTCTGTGT GTGCGGCAGC ATGCGCGGCA GCAAGACAAG
 1901 GAGCGCCTGG CAGCCCTGGG GCCTGAGGGG GCCCATGGTG AACTACCTT
 1951 TGAGTACCAG GACCTGTGCC GCCAGCACAT GGCCACGAAG TCCTTGTTCA
 2001 ACCGGGCAGA GGGTCCACCG GAGCCTTCAC GGGTGAGCAG TGTGTCCTCC
 2051 CAGTTCAGCG ACGCAGCCCA GGCCAGCCCC AGCTCCCACA GCAGCACCCC
 2101 GTCCTGGTGC GAGGAGCCGG CCAAGCCAA CATGGACATC TCCACGGGAC
 2151 ACATGATTCT GGCATACATG GAGGATCACC TGCGBAACCG GGACCGCCTT
 2201 GCCAAGGAGT GGCAGGCCCT CTGTGCCTAC CAAGCAGAGC CAAACACCTG
 2251 TGCCACCGCG CAGGGGGAGG GCAACATCAA AAAGAACCGG CATCCTGACT
 2301 TCCTGCCCTA TGACCATGCC CGCATAAAAC TGAAGGTGGA GAGCAGCCCT
 2351 TCTCGGAGCG ATTACATCAA CGCCAGCCCC ATTATTGAGC ATGACCCTCG
 2401 GATGCCAGCC TACATAGCCA CGCAGGGCCC GCTGTCCCAT ACCATCGCAG
 2451 ACTTCTGGCA GATGGTGTGG GAGAGCGGCT GCACCGTCAT CGTCATGCTG
 2501 ACCCCGCTGG TGGAGGATGG TGTCAAGCAG TGTGACCGCT ACTGGCCAGA
 2551 TGAGGGTGCC TCCCTCTACC ACGTATATGA GGTGAACCTG GTGTGCGAGC
 2601 ACATCTGGTG CGAGGACTTT CTGGTGCGGA GCTTCTACCT GAAGAACGTG
 2651 CAGACCCAGG AGACGCGCAC GCTCACGCAG TTCCACTTCC TCAGCTGGCC

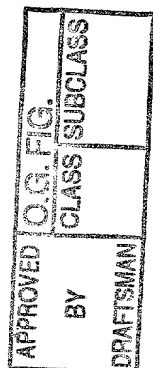


FIG. 3d

055270" 55ESTD60

2701 GGCAGAGGGC ACACCGGCCT CCACGCGGCC CCTGCTGGAC TTCCGCAGGA
 2751 AGGTGAACAA GTGCTACCGG GGCCGCTCCT GCCCCATCAT CGTGCACTGC
 2801 AGTGATGGTG CGGGGAGGAC CGGCACCTAC ATCCTCATCG ACATGGTCCT
 2851 GAACCGCATG GCAAAAGGAG TGAAGGAGAT TGACATCGCT GCCACCCTGG
 2901 AGCATGTCCG TGACCAGCGG CCTGGCCTTG TCCGCTCTAA GGACCAGTTT
 2951 GAATTTGCCC TGACAGCCGT GGCGGAGGAA GTGAATGCCA TCCTCAAGGC
 3001 CCTGCCCCAG TGAGACCCTG GGGCCCCTTG GCGGGCAGCC CAGCCTCTGT
 3051 CCCTCTTTGC CTGTGTGAGC ATCTCTGTGT ACCCACTCCT CACTGCCCCA
 3101 CCAGCCACCT CTTGGGCATG CTCAGCCCTT CCTAGAAGAG TCAGGAAGGG
 3151 AAAGCCAGAA GGGGCACGCC TGCCCAGCCT CGCATGCCAG AGCCTGGGGC
 3201 ATCCCAGAGC CCAGGGCATC CCATGGGGGT GCTGCAGCCA GGAGGAGAGG
 3251 AAAGGACATG GGTAGCAATT CTACCCAGAG CTTTCTCCTG CCTACATTCC
 3301 CTGGCCTGGC TCTCCTGTAG CTCTCCTGGG GTTCTGGGAG TTCCCTGAAC
 3351 ATCTGTGTGT GTCCCCCTAT GCTCCAGTAT GGAAGAATGG GGTGGAGGGT
 3401 CGCCACACCC GGCTCCCCCT GCTTCTCAGC CCCGGGCCTG CCTCTGACTC
 3451 AACTTGGGC GCTCTGCCCT CCCTGGCCTC ACGCCCAGCC TGGTCCCACC
 3501 ACCCTCCCAC CATGCGCTGC TCAACCTCTC TCCTTCTGGC GCAAGAGAAC
 3551 ATTTCTAGAA AAAACTACTT TTGTACCAGT GTGAATAAAG TTAGTGTGTT
 3601 GTCTGTGCAG CTG

FIG. 3e



865270" 86527050

PREPROINSULINI

Exon sequences, i.e. sequences to be used in the patent are underlined and represent exon sequences.

V00565 Length: 4992 December 18, 1997 17:50 Type: N Check: 9721 ..

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| APPROVED | C.G. FIG. | |
| | CLASS | SUBCLASS |
| BY | | |
| DRAFTSMAN | | |

866270-6527060

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1  CTCGAGGGGC CTAGACATTG CCCTCCAGAG AGAGCACCCA ACACCCTCCA
51  GGCTTGACCG GCCAGGGTGT CCCCTTCCTA CCTTGGAGAG AGCAGCCCCA
101 GGGCATCCTG CAGGGGGTGC TGGGACACCA GCTGGCCTTC AAGGTCTCTG
151 CCTCCCTCCA GCCACCCAC TACACGCTGC TGGGATCCTG GATCTCAGCT
201 CCCTGGCCGA CAACACTGGC AAATCCTAC TCATCCACGA AGGCCCTCCT
251 GGGCATGGTG GTCCTTCCCA GCCTGGCAGT CTGTTCTCA CACACCTGT
301 TAGTGCCAG CCCCTGAGGT TGCAGCTGGG GGTGTCTCTG AAGGGCTGTG
351 AGCCCCAGG AAGCCCTGGG GAAGTGCCTG CCTTGCCTCC CCCCAGCCCT
401 GCCAGCGCCT GGCTCTGCCC TCCTACCTGG GCTCCCCCA TCCAGCCTCC
451 CTCCCTACAC ACTCCTCTCA AGGAGGCACC CATGTCCTCT CCAGCTGCCG
501 GGCCTCAGAG CACTGTGGCG TCCTGGGGCA GCCACCGCAT GTCCTGCTGT
551 GGCATGGCTC AGGGTGAAA GGGCGGAAGG GAGGGGTCCT GCAGATAGCT
601 GGTGCCCACT ACCAAACCCG CTCGGGGCAG GAGAGCCAAA GGCTGGGTGT
651 GTGCAGAGCG GCCCCGAGAG GTTCCGAGGC TGAGGCCAGG GTGGGACATA
701 GGGATGCGAG GGGCCGGGGC ACAGGATACT CCAACCTGCC TGGCCCCATG
751 GTCTCATCCT CTTGCTTCTG GGACCTCTG ATCCTGCCCC TGGTGCTAAG
801 AGGCAGGTAA GGGGCTGCAG GCAGCAGGGC TCGGAGCCCA TGCCCCCTCA
851 CCATGGGTCA GGCTGGACCT CCAGGTGCCT GTTCTGGGGA GCTGGGAGGG
901 CCGGAGGGGT GTACCCAGG GGCTCAGCCC AGATGACACT ATGGGGGTGA
951 TGGTGTCATG GGACCTGGCC AGGAGAGGGG AGATGGGCTC CCAGAAGAGG
1001 AGTGGGGGCT GAGAGGGTGC CTGGGGGGCC AGGACGGAGC TGGGCCAGTG
1051 CACAGCTTCC CACACCTGCC CACCCCAGA GTCCTGCCGC CACCCCAGA
1101 TCACACGGAA GATGAGGTCC GAGTGGCCTG CTGAGGACTT GCTGCTTGTC
1151 CCCAGGTCCC CAGGTCATGC CCTCCTTCTG CCACCCTGGG GAGCTGAGGG
1201 CCTCAGCTGG GGCTGCTGTC CTAAGGCAGG GTGGGAACTA GGCAGCCAGC
1251 AGGGAGGGGA CCCCTCCCTC ACTCCACTC TCCCACCCC ACCACCTTGG
1301 CCCATCCATG GCGGCATCTT GGGCCATCCG GGAAGGGGA CAGGGGTCTT
1351 GGGGACAGGG GTCCGGGGAC AGGGTCCTGG GGACAGGGGT GTGGGGACAG

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FIG. 3f

2901 TTCTCCACCC TCATTTGATG ACCGCAGATT CAAGTGTTTT GTTAAGTAAA
2951 GTCCTGGGTG ACCTGGGGTC ACAGGGTGCC CCACGCTGCC TGCCTCTGGG
3001 CGAACACCCC ATCACGCCCC GAGGAGGGCG TGGCTGCCTG CCTGAGTGGG
3051 CCAGACCCCT GTCGCCAGCC TCACGGCAGC TCCATAGTCA GGAGATGGGG
3101 AAGATGCTGG GGACAGGCCC TGGGGAGAAG TACTGGGATC ACCTGTTCAG
3151 GCTCCCACTG TGACGCTGCC CCGGGGCGGG GGAAGGAGGT GGGACATGTG
3201 GGC GTTGGGG CCTGTAGGTC CACACCCAGT GTGGGTGACC CTCCCTCTAA
3251 CCTGGGTCCA GCCCGGCTGG AGATGGGTGG GAGTGCAGACC TAGGGCTGGG
3301 GGGCAGGCGG GCACTGTGTC TCCCTGACTG TGTCTCCTG TGTCCCTCTG
3351 CCTCGCCGCT GTTCCGGAAC CTGCTCTGCG CGGCACGTCC TGGCAGTGGG
3401 GCAGGTGGAG CTGGGCGGGG GCCCTGGTGC AGGCAGCCTG CAGCCCTTGG
3451 CCCTGGAGGG GTCCCTGCAG AAGCGTGGCA TTGTGGAACA ATGCTGTACC
3501 AGCATCTGCT CCCTCTACCA GCTGGAGAAC TACTGCAACT AGACGCAGCC
3551 TGCAGGCAGC CCCACACCCG CCGCCTCCTG CACCGAGAGA GATGGAATAA
3601 AGCCCTTGAA CCAGCCCTGC TGTGCCGTCT GTGTGTCTTG GGGGCCCTGG
3651 GCCAAGCCCC ACTTCCCGGC ACTGTTGTGA GCCCCTCCCA GCTCTCTCCA
3701 CGCTCTCTGG GTGCCACAG GTGCCAACGC CAGGCAGGCC CAGCATGCAG
3751 TGGCTCTCCC CAAAGCGGCC ATGCCTGTTG GCTGCCTGCT GCCCCACCC
3801 TGTGGCTCAG GGTCCAGTAT GGGAGCTTCG GGGGTCTCTG AGGGGCCAGG
3851 GATGGTGGGG CCACTGAGAA GTGACTCTGT CAGTAGCCGA CCTGGAGTCC
3901 CCAGAGACCT TGTTCAGGAA AGGGAATGAG AACATTCCAG CAATTTTCCC
3951 CCCACCTAGC CCTCCCAGGT TCTATTTTGA GAGTTATTTC TGATGGAGTC
4001 CCTGTGGAGG GAGGAGGCTG GGCTGAGGGA GGGGGTCCTG CAGGGCGGGG
4051 GGCTGGGAAG GTGGGGAGAG GCTGCCGAGA GCCACCCGCT ATCCCCAGCT
4101 CTGGGCAGCC CCGGGACAGT CACACACCCT GGCCTCGCGG CCAAGCTGG
4151 CAGCCGTCTG CAGCCACAGC TTATGCCAGC CCAGGTCCAG CCAGACACCT
4201 GAGGGACCCA CTGGTGCCTT GGAGGAAGCA GGAGAGGTCA GATGGCACCA
4251 TGAGCTGGGG CAGGTGCAGG GACCGTGGCA GCACCTGGCA GGGCCTCAGA
4301 ACCCATGCCT TGGGCACCCC GGCCATGAGG CCCTGAGGAT TGCAGCCCAA
4351 GAGAAGCAGG GAACGCCAGG GCCACAGGGG CAGAGACCAG GCCAGGGTCC

| | |
|----------|----------------|
| APPROVED | O.G. FIG. |
| | CLASS SUBCLASS |
| BY | DRAFTSMAN |

FIG. 3h

FIG. 3h

4401 CTTGCGGCCC TTAGCCCACC CCTCCCAGT AAGCAGGGGC TGCTTGGCTA
 4451 GGCTTCCTTT TGCTACAGAC CTGCTGCTCA CCCAGAGGCC CACGGGGCCCT
 4501 AGTGACAAGG TCGTTGTGGC TCCAGGTCCT TGGGGGTCCT GACACAGAGC
 4551 CTCTTCTGCA GCACCCCTGA GGACAGGGTG CTCCGCTGGG CACCCAGCCT
 4601 AGTGGGCAGA CGAGAACCTA GGGGCTGCCT GGGCCTACTG TGGCCTGGGA
 4651 GGTGAGCGGG TGACCCTAGC TACCCTGTGG CTGGGCCAGT CTGCCTGCCA
 4701 CCCAGGCCAA ACCAATCTGC ACCTTTCCTG AGAGCTCCAC CCAGGGCTGG
 4751 GCTGGGGATG GCTGGGCCTG GGGCTGGCAT GGGCTGTGGC TGCAGACCAC
 4801 TGCCAGCTTG GGCCTCGAGG CCAGGAGCTC ACCCTCCAGC TGCCCCGCCT
 4851 CCAGAGTGGG GGCCAGGGCT GGGCAGGCGG GTGGACGGCC GGACACTGGC
 4901 CCCGGAAGAG GAGGGAGGCG GTGGCTGGGA TCGGCAGCAG CCGTCCATGG
 4951 GAACACCCAG CCGGCCCCAC TCGCACGGGT AGAGACAGGC GC

FIG. 3i

| | |
|----------|----------------|
| APPROVED | O.G. FIG. |
| | CLASS SUBCLASS |
| BY | DRAFTSMAN |

1365270" 65EST060

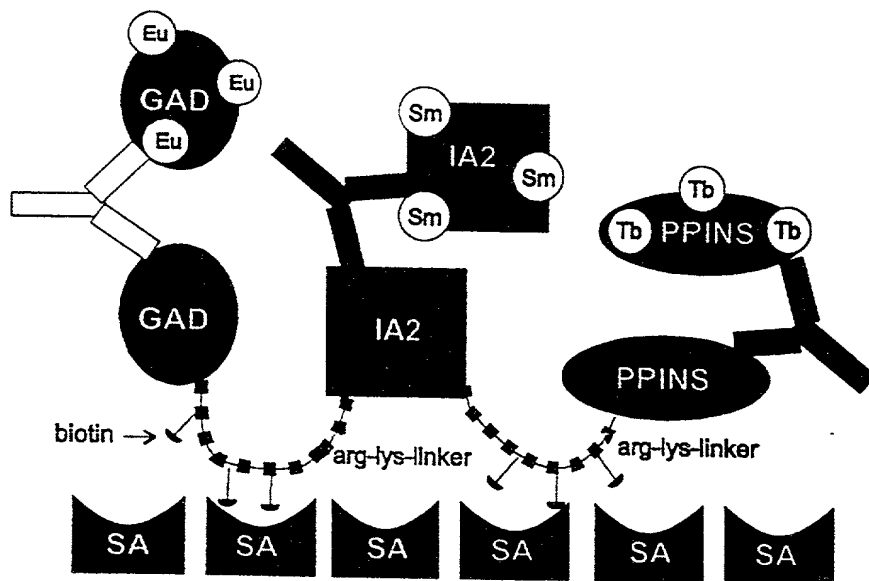


FIG. 4

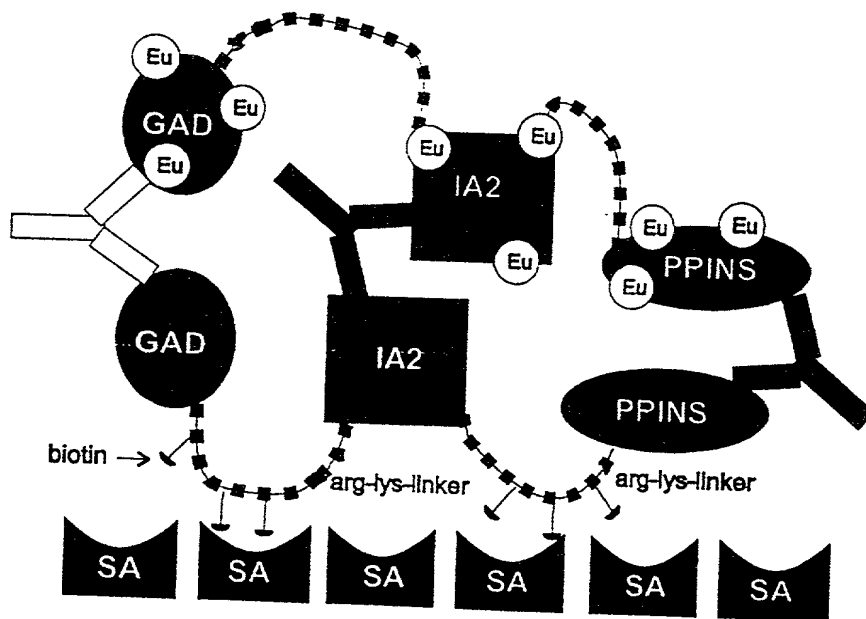


FIG. 5